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PATENT AND TRADEMARK OFFICEATTORNEY DOCKET NO.
P564-9005TRANSMITTAL LETTER TO THE UNITED STATES
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CONCERNING A FILING UNDER 35 U.S.C. 371

DATE: February 17, 1999

U.S. APPLN. NO.
(IF KNOWN, SEE 37 CFR 1.5)

09/147693

INTERNATIONAL APPLICATION NO.
PCT/EP97/04560 ✓INTERNATIONAL FILING DATE
21 August 1997 ✓PRIORITY DATE CLAIMED
21 August 1996 ✓

TITLE OF INVENTION: NEW SYSTEMS FOR THE REGULATION OF GENE EXPRESSION

APPLICANT(S) FOR DO/EO/US: Werner LUBITZ, Wolfgang JECHLINGER, Michael SZOSTAK, Angela WITTE

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
(THE BASIC FILING FEE IS ATTACHED)
 2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
 3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT articles 22 and 39(1).
 4. ☒ A proper demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
 5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
 6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
 7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
 8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
 9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
 10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).
- Items 11. to 16. below concern other document(s) or information included:
11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
 12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
 13. ☐ A FIRST preliminary amendment.
☐ A SECOND or SUBSEQUENT preliminary amendment.
 14. ☐ A substitute specification.
 15. ☐ A change of power of attorney and/or address letter.
 16. ☒ Other items or information: Small Entity Declaration, International Search Report, PCT/RO/101, PCT/IPEA/416, PCT/IPEA/409, PCT/IB/306
CHECK NO. 1877 /
Drawings-5 sheets

APPLN. NO. (IF KNOWN, SEE 37
C.F.R. 1.50)

INTERNATIONAL APPLICATION
NO. PCT/EP97/04560

ATTORNEY DOCKET NO.: P564-9005

DATE: February 17, 1999

17. XX The following fees are submitted:

Basic National Fee (37 CFR 1.492(a)(1)-(5):

Search Report has been prepared by the EPO or JPO.....\$840.00
International preliminary examination fee paid to USPTO (37 CFR 1.482).....\$670.00
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but
international search fee paid to USPTO (37 CFR 1.445(a)(2)).....\$760.00
Neither international preliminary examination fee (37 CFR 1.482) or international
search fee (37 CFR 1.445(a)(2)) paid to USPTO.....\$970.00
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all
claims satisfied provisions of PCT Article 33(2)-(4)\$ 96.00

CALCULATIONS

PTO USE ONLY

ENTER APPROPRIATE BASIC FEE AMOUNT =

\$840

Surcharge of \$130.00 for furnishing the oath or declaration later than _ 20 _ 30
months from the earliest claimed priority date (37 CFR 1.492(e)).

\$00

Claims

Number Filed

Number Extra

Rate

Total Claims

40 - 20 =

20

X \$ 18.00

\$360

Independent Claims

03 - 3 =

00

X \$ 78.00

\$00

Multiple dependent claim(s) (if applicable)

+ \$260.00

\$260

TOTAL OF ABOVE CALCULATIONS =

\$1,460

Reduction by 1/2 for filing by small entity, if applicable.
Verified Small Entity statement must also be filed.
(Note 37 CFR 1.9, 1.27, 1.28).

\$730

SUBTOTAL =

\$730

Processing fee of \$130.00 for furnishing the English translation later the _ 20 _ 30
months from the earliest claimed priority date (37 CFR 1.492(f)).

\$00

TOTAL NATIONAL FEE =

\$730

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must
be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per
property

\$40

TOTAL FEES ENCLOSED =

\$770

Amount to be refunded

\$

Charged

\$

a. XX A check in the amount of \$770 to cover the above fees is enclosed.

b. _ Please charge my Deposit Account No. 14-1060 in the amount of \$_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. XX The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1060.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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10

Serial or Patent No.: _____ Docket No.: _____

Filed or Issued: _____

To: _____

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(c) - SMALL BUSINESS CONCERN

I hereby declare that I am

- () the owner of the small business concern identified below:
() an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN EVAX Technologies AG

ADDRESS OF CONCERN Fraunhoferstraße 10, D-82152 Martinsried/München, Germany

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled New systems for the regulation of gene expression by Inventor(s) Werner Lubitz, Wolfgang Jechlinger, Michael Szostak and Angela Witte described in

- () the specification filed herewith
() application serial no. _____ filed _____
() patent no. _____, issued _____

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e). NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME _____

ADDRESS _____
() INDIVIDUAL () SMALL BUSINESS CONCERN () NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28 (b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING DR. MARTIN STEINER

TITLE OF PERSON OTHER THAN OWNER CEO

ADDRESS OF PERSON SIGNING FRAUNHOFERSTRASSE 10 D-82152 MARTINSRIED

SIGNATURE [Signature] DATE 3. Feb 1999

New systems for the regulation of gene expression

Description

The present invention concerns a method for selecting new P_R or P_L operator sequences from lambdoid phages which have a different thermostability compared to the wild-type sequence with regard to binding a repressor. In addition new mutated P_R or P_L operator sequences and their application for the temperature-regulated expression of genes and for the production of improved vaccines are disclosed.

The initiation of transcription of the O_R - O_L region of the bacteriophage lambda and other lambdoid phages is negatively and positively regulated by a repressor which is the product of the cI gene (see review article Ptashne et al., Cell 19 (1980), 1-11). In the O_R region three operator sequences (O_{R1} , O_{R2} and O_{R3}) overlap the promoters P_R and P_{RM} which are orientated in different directions. P_R controls the transcription of genes which are responsible for the lytic multiplication cycle of the phage whereas P_{RM} is the promoter for the lambda cI gene which is responsible for maintaining the lysogenic state. The lambda repressor cI binds co-operatively to the operator sequences O_{R1} and O_{R2} with the result that P_R is repressed and P_{RM} is activated.

In addition the bacteriophage lambda also contains a further operator region O_L which also contains three operator sequences (O_{L1} , O_{L2} and O_{L3}). The expression of the lambda N gene can be repressed by the P_L promoter by binding of the cI repressor to this O_L operator region.

Promoters of the bacteriophage lambda in particular the P_L and the P_R promoter have been used for a long time in recombinant DNA technology for heterologous temperature-regulated gene expression in E.coli (cf. Hedgpeth et al., Molec.Gen.Genet. 183 (1978), 197-203 and Bernard et al., Gene 5 (1979), 59-76; Buell et al., Nucleic Acids Res. 13 (1985), 1923 and Shatzman and Rosenberg, Methods Enzymol. 152 (1987), 661). A temperature-sensitive lambda repressor $cI857$ is used in these expression systems which represses the P_L and P_R transcription at low temperatures up to 30°C but allows a gene expression at higher temperatures.

An advantage of this lambda expression system is that the gene expression can be induced in a simple manner by increasing the temperature and no addition of chemical inducers is necessary for this. However, a serious disadvantage is that the repression of gene expression only occurs up to relatively low temperatures of not more than 30°C, which is a temperature at which only a slow bacterial growth occurs. Hence the object of the invention was to provide an improved system for lambda P_L or P_R gene expression which enables a repression at variable higher temperatures.

This object is achieved by providing mutated P_R or P_L operator sequences from lambdoid phages which, compared to the wild-type operator sequence, have a different and in particular higher thermostability with regard to the binding of a temperature-sensitive repressor. The finding that lambda expression systems with an improved thermostability can be produced at all is extremely surprising since, apart from the temperature-sensitive lambda $cI857$ mutant, no other temperature-sensitive cI mutants are known but only those mutations in the cI

repressor are known which make the molecule more resistant to thermal inactivation (Hecht et al., Proteins 1 (1986), 43-46 and Das and Mandal, Mol.Gen.Genet. 204 (1986), 540-542). It was even more surprising that mutations which lead to an improved thermostability are located in the operator DNA sequence and not in the DNA sequence coding for the repressor molecule. Thus for example a mutation of the lambda O_R2 operator sequence is known from the literature which leads to a complete loss of repressor binding (Hawley et al., J.Biol.Chem. 260 (1985), 8618-8626).

A method is provided for identifying suitable mutants which enables the selection of mutated O_R or O_L operator DNA sequences from lambdoid phages which have a different thermostability compared to the wild-type sequence with regard to binding a repressor in which the method is characterized in that (a) a DNA cassette is prepared which contains a selection gene under the operative control of an expression control sequence comprising at least one O_R or O_L operator sequence from a lambdoid phage and a promoter, (b) the operator DNA sequence is subjected to a mutagenesis and (c) the mutated operator DNA sequences are analysed.

The lambdoid phages are preferably selected from the group comprising the phage lambda, phage 21, phage 22, phage 82, phage 424, phage 434, phage D326, phage DLP12, phage gamma, phage HK022, phage P4, phage Phi80, phage Phi81, coliphage 186 and recombinant variants thereof. The said phages are very similar with regard to the mechanism of repression of gene expression by means of a cI repressor (Johnson et al., Nature 294 (1982), 217-223). Recombinant variants of the said phages e.g. lambda imm434 can be obtained by substitution of

individual genome fragments within the said phages (cf. for this Hendricks et al., Lambda 2 (1983), R.W. Hendricks, J.W. Roberts, F.W. Stahl and R.A. Weissberg (publisher), Cold Spring Harbor Laboratory Press, New York). The phage lambda or a recombinant variant thereof is preferably used as the lambdoid phage e.g. lambda imm434. An operator DNA sequence from the operator regions O_R (SEQ ID NO.1) or /and O_L (SEQ ID NO.3) of the phage lambda and in particular one of the operator sequences O_{R1} , O_{R2} and O_{R3} or O_{L1} , O_{L2} and O_{L3} contained therein is particularly preferably used for the mutagenesis. The operator sequence O_{R2} is most preferred.

The selection gene for the DNA cassette which is brought under the operative control of the expression control sequence containing the mutated operator sequence, preferably a lambda operator/promoter region, is preferably a suicide gene which when expressed leads to the death of the bacterial cell and thus serves as a selection marker for identifying suitable mutants. The suicide gene should be so strongly repressed at a temperature at which the lambda repressor binds to the mutated operator sequence that a bacterial cell containing the DNA cassette can grow. When the maximum temperature at which the repressor can still bind to the operator is exceeded, the suicide gene is expressed and the bacterial cell is destroyed. This enables a simple and direct selection of suitable mutated operator sequences. A suitable suicide gene is the E lysis gene from the phage PhiX174 as well as homologues and derivatives derived therefrom (Hutchison and Sinsheimer, J.Mol.Biol. 18 (1966), 429-447; Witte et al., Multifunctional safety vector systems for DNA cloning, controlled expression of fusion genes and

simplified preparation of vector DNA and recombinant gene products, in BioTech Forum, Advances in Molecular Genetics 3, pp 219-239, publisher: Issinger, O.-G., Henke, J., Kämpf, J., Driesel, A.J., Hüthing Verlag 1991, Heidelberg). Further examples of suitable lysis genes are GEF (Poulsen et al., Mol.Microbiol. 5 (1991), 1627-1637) and Kil (Reisinger et al., Virology 193 (1993), 1033-1036). On the other hand the selection gene can also be a reporter gene such as e.g. the β -Gal gene.

In order to produce mutants the operator DNA sequence is preferably subjected to a site-specific mutagenesis using one or several oligonucleotides for example according to the method of Kunkel (Proc.Natl.Acad.Sci. USA 82 (1985), 488-492) or they are obtained by selection in a mutator bacterial strain e.g. an E. coli mutD or mutL mutator strain such as E. coli ES1578 (Wu et al., Gene 87 (1990), 1-5). The mutated operator DNA sequences are preferably selected by determining the ability to bind to a temperature-sensitive cI repressor in particular to the temperature-sensitive cI857 repressor. For this the DNA cassette which is preferably located on a vector is transformed into a bacterial cell which contains a gene coding for a temperature-sensitive cI repressor. This gene may also be present on a vector (Remaut et al., Gene 15 (1981), 81-93). On the other hand it is possible to use a bacterial cell which contains such a repressor gene in its chromosome e.g. E. coli M5219 (cf. e.g. Shimatake and Rosenberg, Nature 292 (1981), 128).

Mutants which are resistant to lysis at different temperatures can be identified in a simple manner by culturing the bacterial cells transformed with a lysis cassette which contain the mutated operator DNA

sequences. Up to now it has been possible to identify several mutants which are resistant to a lysis at temperatures up to 33°C, 35°C, 37°C and 39°C. These bacteria contain mutated operator DNA sequences which allow binding of the repressor up to the respective temperature. A particularly preferred example is a mutant to which the cI857 repressor binds up to a temperature of about 37°C. Compared to the wild-type the mutation is a single base substitution in the O_R2 section of the lambda O_R operator region. The sequence of this mutated lambda O_R operator is shown in SEQ ID NO.2.

An additional subject matter of the invention are mutated O_R or O_L operator sequences from lambdoid phages which have a different thermostability compared to the wild-type sequence with regard to binding of a repressor and which are obtainable by the selection methods described above. The mutated O_R or O_L operator sequences preferably have an increased thermostability with regard to the binding of a temperature-sensitive repressor and in particular of the temperature-sensitive cI repressor. The mutated operator sequences particularly preferably have a thermostability that is increased by about 3 to 10°C, in particular by about 7 to 9°C compared to the wild-type sequence.

Since the selection method according to the invention is preferably carried out on O_R or O_L operator sequences which are derived from the phage lambda, the present invention in particular concerns mutated lambda O_R or O_L operator sequences which are variants of the O_R operator sequences shown in SEQ ID NO.1 or variants of the O_L operator sequences shown in SEQ ID NO.3. Variant in this connection is understood as an operator sequence which differs from the wild-type sequence in at least one

sequence position by insertion, deletion or substitution of bases. The differences are particularly preferably in the region of the sections O_R1 , O_R2 or O_R3 and O_L1 , O_L2 and O_L3 . A specific example of a mutated lambda operator sequence according to the invention is the lambda O_R operator sequence shown in SEQ ID NO.2.

The mutated operator sequences allow the production of new temperature-regulated systems for gene expression in which microorganisms and in particular bacteria can be cultured in a repressed state at variable temperatures and preferably at higher temperatures than have been previously possible in particular at 33 to 39°C. Hence a subject matter of the invention is the use of the mutated O_R or O_L operator sequences for the temperature-regulated expression of genes in bacteria and in particular in gram-negative bacteria such as *E. coli*. Combination of a wild-type O_R or O_L operator region and at least one operator region which contains a mutated operator sequence according to the invention or combination of several operator regions which contain mutated operator sequences according to the invention with different thermostabilities even enables a temperature-regulated sequential expression of genes.

Vectors and bacterial strains in which the inventive mutated operator sequences can be used for the temperature-regulated expression of genes are familiar to a person skilled in the art. In this case the expression systems known from the prior art containing the lambda cI857 repressor in combination with a suitable promoter e.g. the lambda P_L or lambda P_R promoter can be used (cf. e.g. Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd Edition, 1989, Cold Spring Harbor Laboratory Press, New York,

17.11-17.12).

A further subject matter of the present invention is a nucleic acid comprising a bacterial expression control sequence i.e. a sequence containing a promoter and operator regions which contains a mutated O_R or O_L operator sequence according to the invention in operative linkage with a protein-coding sequence. The protein-coding sequence can for example be a sequence coding for a eukaryotic protein or polypeptide or a bacterial gene e.g. the E-lysis gene.

An additional subject matter of the present invention is a vector which contains at least one copy of the bacterial expression control sequence in operative linkage with the protein-coding sequence. This vector can be any prokaryotic vector e.g. a chromosomal vector such as a bacteriophage or an extrachromosomal vector such as a plasmid. Suitable prokaryotic vectors are described for example by Sambrook et al., Supra, chapters 1-4.

Yet a further subject matter of the present invention is a bacterial cell which is transformed with a nucleic acid according to the invention or with a vector according to the invention. In a preferred embodiment the cell is a gram-negative prokaryotic cell, particularly preferably an E. coli cell. The cell preferably contains the nucleic acid or the vector integrated into its chromosome and in addition contains a gene for a cI repressor from a lambdoid phage in particular the gene for the lambda cI857 repressor.

A particularly preferred application of the mutated

operators according to the invention is in the field of vaccine production. So-called "bacterial ghosts" are known as vaccines from the prior art i.e. bacterial coats that can be prepared from gram-negative bacteria such as *E. coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Actinobacillus pleuropneumoniae* etc. by means of protein-E-induced lysis. These ghosts whose cell surface properties and repertoire of surface antigens that can be recognized by the immune system are very similar to the active pathogen, produce a protective cellular or/and humoral immune response in various animal models.

The process for preparing the ghosts is based on the stringent controlled expression of the E-lysis gene from Φ IX174 whose expression product forms a tunnel through the bacterial cell wall coat and thus leads to a pouring out of the cell contents of the host cell. This lethal gene for the cells can be regulated by means of a lambda repressor e.g. the temperature-sensitive lambda repressor cI857 which, as described above, loses its function at temperatures above 30°C. As a result, the bacterial cultures that have previously been used to produce bacterial ghosts have had to be cultured at low temperatures, preferably at 28°C.

Although this method leads to satisfactory results with regard to the immunogenicity of the ghosts that are produced, an improvement of the bacterial culture is urgently required since the repertoire of antigenic determinants on the bacterial surface can change depending on the external conditions. Since pathogenic bacteria which infect humans or animals usually colonize at an environmental temperature of 37 to 39°C, this natural environmental temperature should also be

maintained during the production process for the ghosts.

A process for producing bacterial ghosts which achieves this object is provided by using the mutated operator sequences according to the invention. These operator sequences allow growth of the bacteria up to a temperature range of preferably 35 to 39°C and allow lysis when the temperature is increased from 37 to 42°C. This changed lysis behaviour enables the pathogens to be cultured near to the body temperature of the vaccine candidate which is extremely important for the composition of the external membrane. Furthermore the new lysis cassette can also be used as a safety cassette in live vaccines since for example in humans the inoculated bacteria are killed when fever is induced (39°C).

Hence a subject matter of the invention is a vaccine composition which contains a live bacterial cell according to the invention as the active ingredient optionally together with pharmaceutically tolerated auxiliary substances, additives and carrier substances. The live bacterial cell contains a nucleic acid comprising a bacterial expression control sequence with a mutated operator sequence in operative linkage preferably with a lysis gene. Yet a further subject matter of the present invention is a vaccine composition which contains a bacterial ghost as an active ingredient optionally together with pharmaceutically tolerated auxiliary substances, additives and carrier substances wherein the bacterial ghost is obtainable by culturing a bacterial cell according to the invention at temperatures of 35 - 39°C and subsequently lysing the bacterial cell by increasing the temperature. Bacterial cells suitable as vaccines are in particular gram-

negative bacteria such as *E. coli* for example the strains STEC, EHEC, O78:K80, *Salmonellae* such as *S. choleraesuis*, *S. enteritidis* and *S. typhimurium*, *Pasteurella multocida*, *Pasteurella haemolytica*, *Bordetella bronchiseptica*, *Klebsiella pneumoniae*, *Actinobacillus pleuropneumoniae*, *Haemophilus influenzae*, *Vibrio cholerae*, *Helicobacter pylori*, *Alcaligenes eutrophus*, *Campylobacter jejuni* and *Pseudomonas aeruginosa*.

The vaccine compositions modified according to the invention can be transferred orally, aerogenically or parenterally to the vaccine candidates. The route which the corresponding microorganisms naturally select for the infection and for the initial stages of establishing an infectious disease are preferably selected for the application of the vaccine. Since all surface properties are retained in the vaccines according to the invention, this application can result in a local induction of the immune response as also occurs in the natural infection process.

As described above the use of mutated operator sequences according to the invention enables the development of vaccines that can be lysed in a controlled manner when a target temperature is exceeded. Furthermore it is, however, also possible to provide a cold-sensitive suicide cassette which on release into the environment kills gram-negative bacteria that are used as a live vaccine. Hence combination of two genetic regulation systems enables the bacteria to die as a result of the expression of a suicide gene when a target value for the environmental temperature is exceeded. This safety cassette ensures that the live vaccines are killed even if they are eliminated from the organisms.

Hence the invention concerns a nucleic acid comprising (a) a first bacterial expression control sequence which contains an O_R or O_L operator sequence from a lambdoid phage and to which a first temperature-sensitive cI repressor from lambdoid phages can bind in operative linkage with a sequence coding for a second repressor wherein the second repressor cannot bind to the first bacterial expression sequence and (b) a second bacterial expression control sequence to which the second repressor can bind which is in operative linkage with a suicide gene.

The components (a) and (b) can be covalently linked together e.g. be present on a single vector or be separate from one another e.g. present on different vectors or be located separately or together on the chromosome of a recipient bacterium.

Yet a further subject matter of the present invention is a bacterial cell which contains at least one copy of a nucleic acid as described above. In addition the bacterial cell advantageously contains a gene for the first repressor. The first repressor is preferably the temperature-sensitive cI857 repressor.

The safety cassette according to the invention preferably contains a gene which codes for a temperature-sensitive cI repressor e.g. the repressor cI857 and a gene which codes for a second repressor wherein this gene is under the control of a lambda promoter/operator region to which the temperature-sensitive repressor binds. The second repressor in turn controls the expression of another gene e.g. a suicide gene such as the E-lysis gene. The temperature-sensitive

lambda repressor is inactive at 37°C so that the second repressor is expressed which in turn represses the expression of the suicide gene.

When the temperature is reduced the temperature-sensitive lambda repressor binds to the operator so that the expression of the second repressor is blocked which leads to an expression of the suicide gene. A first expression control sequence is preferred for this safety cassette which contains the mutated lambda operator since this enables an improved and more rapid activation of the suicide gene.

The second repressor can be any repressor e.g. a lac repressor. However, it is preferable to use an additional repressor from lambdoid phages e.g. cI from the phage 434 which is not temperature-sensitive and binds to its own operator sequence but does not bind to the sequence recognized by the lambda repressor cI857.

It is particularly preferable for the development of live vaccines to incorporate a heat as well as a cold regulation element. This incorporation is preferably achieved by homologous recombination into the chromosome of the vaccine bacterium.

Thus the present invention also concerns a bacterial cell which, in addition to the two components (a) and (b), contains a third bacterial expression control sequence as component (c) which contains a mutated operator sequence according to the invention in operative linkage with a suicide gene.

These bacterial cells can also be used in vaccine compositions and especially for live vaccines. In this manner it is possible to produce heat or/and cold-sensitive safe live vaccines which lead to death of the vaccine bacteria when the body temperature of the vaccine candidate is increased e.g. by fever or/and when they are excreted into the environment.

It is intended to additionally elucidate the invention by the following figures, sequence protocols and examples.

- Fig. 1a shows a schematic representation of a lysis cassette of the prior art comprising a lambda O_R wild-type region, the lambda cI857 gene under the control of the promoter P_{RM} and the E lysis gene under the control of the promoter P_R ;
- Fig. 1b shows a schematic representation of a lysis cassette according to the invention which contains a mutated lambda O_R sequence;
- Fig. 2a shows a schematic representation of a cold-sensitive safety cassette comprising a wild-type (pCS1) or mutated (pCSJ1) O_R operator sequence, the lambda-cI857 gene under the control of the promoter P_{RM} , the gene of the lacI repressor under the control of P_R and the E-lysis gene under the control of the lac promoter/operator system at a temperature at which the temperature-sensitive lambda repressor cI857 does not bind to the lambda O_R sequence;
- Fig. 2b shows a schematic representation of the safety cassette according to Fig. 2a at a temperature at which the lambda repressor cI857 binds to the lambda O_R operator;

- Fig. 3 shows the lysis curve of bacterial cells (optical density versus time) which contain a plasmid with the lysis cassette shown in Fig. 1b;
- Fig. 4 shows the lysis curve of a bacterial cell which contains a cold-sensitive safety cassette with the wild-type O_R operator and
- Fig. 5 shows a comparison of lysis curves of bacterial cells which contain a cold-sensitive safety lysis cassette with the wild-type O_R operator (pSC1) or the mutated operator (pCSJ1);
- Fig. 6a shows a schematic representation of a cold-sensitive safety cassette comprising a wild-type (pCS2) or mutated (pCSJ2) O_R operator sequence, the lambda cI857 gene under the control of the promoter P_{RM} , the gene of the phage 434 cI repressor under the control of lambda P_R and the E lysis gene under the control of the 434 O_R (P_{RM} - P_R) promoter/operator system at a temperature at which the temperature-sensitive lambda repressor cI857 does not bind to the lambda O_R sequence,
- Fig. 6b shows a schematic representation of the safety cassette according to Fig. 6a at a temperature at which the lambda repressor cI857 binds to the lambda O_R operator;

SEQ ID NO.1 shows the nucleotide sequence of the lambda O_R operator; the operator sequence O_{R3} extends from position 11 - 27; the operator sequence O_{R2} extends from position 34 - 41; the operator sequence O_{R1} extends from position 58 - 74;

SEQ ID NO.2 shows the nucleotide sequence of a mutated lambda O_R operator which, compared to the wild-type sequence, has a substitution of

T → C at position 42;

SEQ ID NO.3 shows the nucleotide sequence of the lambda O_L operator; the operator sequence O_L3 extends from position 11 - 27; the operator sequence O_L2 extends from position 31 - 47; the operator sequence O_L1 extends from position 55 - 70:

SEQ ID NO. 4 to 6

show a 1601 bp long DNA fragment of the plasmid pAW12; bp 1 - 983 are derived from the bacteriophage lambda (position 37125 - 38107; cf. Sanger et al., J.Mol.Biol. 162 (1982), 729-773) and contain the lambda cI857 gene (position 816-106; SEQ ID NO.5) as well as the mutated O_R operator region (mutation at position 858 T → C); bp 1023 - 1601 are derived from the phage PhiX174 (position 447 - 1026; cf. Sanger et al., J.Mol.Biol. 125 (1978), 225-246) and contain the E-lysis gene (position 1144 - 1416; SEQ ID NO.6);

SEQ ID NO. 7 to 10

shows a 2834 bp long DNA fragment of the plasmid pCSJ; bp 1 - 983 are derived from the bacteriophage lambda (position 37125 - 38107) and contain the cI857 gene (position 816 - 106; SEQ ID NO.5) as well as the mutated lambda O_R region (mutation at position 858 T → C; bp 990 - 2230 are derived from the E. coli lac operon subcloned on the plasmid pMC7 (Calos, Nature 274 (1978), 762-765) and contain the lacI repressor gene (bp 1025 - 2104; SEQ ID NO.9) and the lac promoter/operator; bp 2256 - 2834 are derived from the bacteriophage

PhiX174 (position 447 - 1026) and contain the E-lysis gene (bp 2377 - 2649; SEQ ID NO.10).

Examples

Example 1:

1.1 Random mutagenesis of the lambda O_R operator region

The plasmid pAW12 (Witte and Lubitz, Eur.J.Biochem. 180 (1989), 393-398) was selected as the starting material which contains the lysis gene E from the bacteriophage PhiX174 under the control of the lambda P_R promoter as well as the associated repressor gene cI857. The aim of this experiment was to change the lysis cassette so that the lysis gene E is not already activated at 30°C but at higher temperatures. For this the E. coli mutator strain ES1578 (Wu et al., (1990), supra) was transformed with the lysis plasmid and a selection was carried out for clones with a changed temperature profile of cell lysis.

For this the mutated clones produced by the transformation were detected after being stamped onto test plates containing lysis selective medium (LB containing 1 % SDS) and incubated at different temperatures (e.g. 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C). The changed lysis profile of the lysis cassette in liquid culture was exactly determined by plasmid extraction and subsequent transformation into a non-mutator test strain.

The type of mutation was determined by subcloning the

mutagenized lysis cassettes into a sequencing plasmid. In addition the lysis gene E was substituted by the β -galactosidase gene for a functional check. It was then possible on the basis of a simple β -gal test to quantitatively measure the repressed or active state of the gene cassette.

In this manner it was possible to obtain several clones with a different temperature lysis profile. These clones allowed growth of the bacteria in a temperature range of 33-39°C and did not lead to lysis of the bacteria until the temperature was further increased to 37-42°C.

A mutation of the O_R operator region (SEQ ID NO.2) was identified by sequencing a mutated clone which had a thermostability up to 37°C.

1.2 Verification of the mutation

In order to verify the mutation obtained in example 1.1. a site-specific mutagenesis of the lambda O_R wild-type sequence was carried out using an oligonucleotide.

The mutagenesis was carried out according to the protocol of Kunkel (Proc.Natl.Acad.Sci. USA 82 (1985), 488-492).

4 ml overnight culture of the E. coli strain CJ236 (dut^- , ung^-) was added to 50 ml LB medium (+ 10 μ g/ml chloramphenicol and 0.25 μ g/ml uridine) and shaken for 30 min at 37°C. Then 100 μ l M13 phages was added and it was incubated for 6 h at 37°C.

The culture was centrifuged in 2 SS34 centrifuge tubes for 10 min at 14000 rpm and 4°C, the supernatant was decanted into new tubes and again centrifuged for further purification.

The phages were precipitated for 1 h at 4°C by addition of 5 ml 5 x polyethylene glycol/NaCl. They were then centrifuged for 10 min at 14000 rpm and 4°C and the supernatant was discarded.

The pellet was dried, suspended in 0.8 ml TES buffer (0.1 M Tris HCl, pH 8; 0.3 M NaCl; 1 mM EDTA) and incubated for 1 h at 4°C. The suspension was divided into 2 Eppendorf vessels and centrifuged for 5 min at 5000 rpm. The supernatant in which the disrupted phages were located was removed and subjected to a phenol/chloroform extraction to isolate the DNA. The resulting DNA was precipitated with a 2.5-fold volume of 96 % ethanol, washed with 70 % ethanol and taken up in 60 μ l H₂O.

An oligonucleotide with the sequence
5'-GTA AAA TAG TCA ACA CGC GCG GTG TTA GAT ATT TAT C-3'
was phosphorylated. For this 20 μ l H₂O, 20 μ l oligonucleotide (20 ng), 4.5 μ l kinase buffer (Stratagene) and 0.5 μ l polynucleotide kinase (5 U, Stratagene) was incubated for 1 h at 37°C. Then 7 μ l 0.1 M EDTA was added and it was heated for 10 min to 65°C.

For the annealing 20 μ l phosphorylated oligonucleotide, 3.5 μ l single-stranded DNA template (1 μ g ssDNA produced as described above) and 1.4 μ l 20 x SSC buffer were heated for 5 min to 70°C, slowly cooled to 25°C and then

placed on ice.

For the extension 10 μ l of the reaction mixture from the annealing mixture, 37.5 μ l XL buffer (27 mM Hepes pH 7.8, 5 mM of each dNTP, 13 mM $MgCl_2$, 2.7 mM dithiothreitol, 1.3 mM ATP, 1 μ l ligase (1 U, Boehringer Mannheim), 1.5 μ l T4 polymerase (1.5 U, Boehringer Mannheim), 1.5 μ l T4 gene32 protein (8 μ g, Boehringer Mannheim) were incubated for 10 min on ice, 10 min at room temperature and 2 h at 37°C. After 1 h 1 μ l ligase and 1 μ l T4 DNA polymerase was added. After completion of the incubation the reaction was stopped by adding 3 μ l 0.25 M EDTA.

For the transformation 100 μ l competent E. coli cells JM103 (Messing et al., Nucleic Acids Res. 9 (1981), 309-321) was admixed with 10 μ l DNA from the extension mixture and incubated for 1 h or more on ice. After a heat shock for 2.5 min at 42°C, 0.2 ml fresh JM103 cells was added in the logarithmic growth phase. The cells were mixed with 3 ml soft agar and inoculated on an LB agar plate. They were subsequently incubated overnight at 37°C.

In order to identify the mutants, plaques were pricked out with a Pasteur pipette and used to inoculate 5 ml LB medium to which 400 μ l of an overnight culture of E. coli JM103 had been added. After 3 h growth at 37°C, the cells were centrifuged. Double-stranded M13 plasmids were obtained from the cell pellet by means of plasmid preparation. Single-stranded M13 phages could be isolated from the supernatant.

Example 2:

Analysis of the mutagenized lysis cassettes

Figures 1 and 2 shows different E-specific lysis cassettes with different temperature inductions of the lysis function.

In Fig. 1a which contains the wild-type lambda O_R operator sequence, the function of the E-lysis gene is repressed up to 30°C by the cI857-coded repressor protein on the preceding lambda P_R promoter/operator region. cI857-specific repressor molecules are thermally inactivated at temperatures above 30°C and the expression of the E gene is induced. Fig. 1b shows the plasmid pAWJ12 which contains a mutated operator sequence (SEQ ID NO.2) so that the repression of the lysis function of the gene E by cI857 occurs up to 37°C and the lysis function is not induced until 39°C or higher temperatures are reached.

The function of a cold-sensitive safety cassette is elucidated in Fig. 2. Fig. 2a shows that the formation of lacI-specific repressor molecules which in turn repress the expression of the E gene is induced in the plasmids pCS1 (wild-type operator) and pCSJ1 (mutated operator) at a temperature of $\geq 32^\circ\text{C}$ (pCS1) or $\geq 39^\circ\text{C}$ (pCSJ1). At a temperature below 30°C (pCS1) or 37°C (pCSJ1) functional cI857 repressor molecules are formed which suppress the formation of lacI-specific repressor molecules and thus allow the expression of the E gene (Fig. 2b). In the plasmid pCSJ1 the concomitant cell lysis occurs more rapidly than in pCS1.

Fig. 3 shows the lysis curve of a bacterial cell containing the plasmid pAWJ12 (mutated operator). 3 hours after beginning the culture, the temperature was maintained at 37°C in an aliquot of the bacterial cells and increased in two other aliquots to 38 and 42°C. At 37°C there was a further growth of the bacteria whereas a lysis already occurred at 38°C. The lysis is considerably increased at 42°C.

Figures 4 and 5 show the function of a cold-sensitive safety cassette. In Fig. 4 bacterial cells which contained the plasmid pCS1 (wild-type operator) were subjected to a temperature change from 37 to 28°C. This reduction in temperature led to the E-lysis gene being switched off and cell death (decrease of the optical density).

Fig. 5 shows a comparison of the lysis rate of bacteria which contain the plasmid pCS1 (wild-type operator) and the plasmid pCSJ1 (mutated operator). It can be seen that the lysis occurs much more rapidly in the bacteria which contain the mutated operator.

Fig. 6 shows a further cold-sensitive safety cassette. At temperatures at which the lambda cI857 repressor does not bind to the operator the plasmids pCS2 (wild-type operator) and pCSJ2 (mutated operator) form cI-434 repressor molecules which repress the expression of the E gene (Fig. 6a). Formation of cI-434-specific repressor molecules is prevented thus allowing expression of the E gene at a temperature at which the cI857 repressor binds to the lambda operator (Fig. 6b).

Example 3:

In vivo analysis of cold-sensitive lysis cassettes

The killing of bacteria by lowering the temperature after passage through a mouse intestine and excretion into the faeces was determined.

For this 10^{10} E. coli bacteria were administered once to Balb/c mice and the excreted number of bacteria in the faeces was determined. The evaluation was carried out on E.coli-specific Endo plates (Endo, "Zentralbl. Bakt. I Orig." 35 (1904) 109-110) using tetracyclin as a marker for the plasmids used. The incubation was carried out at 28°C.

Results:

In the experimental groups E. coli NM522 (pCS2), E. coli MC4100 (pCS1) and E. coli MC4100 (pCSJ1) there was a reduction in the germ count compared to an E. coli NM522 (pAWJ-lac) control of at least 99.9 %, 98 % and 80 % measured 10 h and 20 h after administering the E. coli bacteria.

SEQUENCE PROTOCOL

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Prof.DR. Werner Lubitz
- (B) ROAD: Schoenborngasse 12/7
- (C) CITY: Vienna
- (E) COUNTRY: Austria
- (F) POSTAL CODE: 1080

(ii) TITLE OF INVENTION: New systems for the regulation
of gene expression

(iii) NUMBER OF SEQUENCES: 10

(iv) COMPUTER-READABLE FORM:

- (A) DATA CARRIER: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0,
Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 82 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(vi) INITIAL ORIGIN:

- (A) ORGANISM: lambda OR operator (wild-type)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ACGTTAAATC TATCACCGCA AGGGATAAAT ATCTAACACC GTGCGTGTTG ACTATTTTAC
CTCTGGCGGT GATAATGGTT GC

60

82

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 82 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(vi) INITIAL ORIGIN:
 (A) ORGANISM: lambda OR operator (mutant)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ACGTTAAATC TATCACCGCA AGGGATAAAT ATCTAACACC GCGCGTGTTG ACTATTTTAC	60
CTCTGGCGGT GATAATGGTT GC	82

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 85 base pairs
 (B) TYPE: nucleotide
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: linear

(vi) INITIAL ORIGIN:
 (A) ORGANISM: lambda OL operator (wild-type)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ACATACAGAT AACCATCTGC GGTGATAAAT TATCTCTGGC GGTGTTGACA TAAATACCAC	60
TGGCGGTGAT ACTGAGCACA TCAGC	85

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1601 base pairs
 (B) TYPE: nucleotide
 (C) STRANDEDNESS: double strand
 (D) TOPOLOGY: both

(vi) INITIAL ORIGIN:
 (A) ORGANISM: pAW12 fragment

(xi) CHARACTERISTICS:
 (A) NAME/KEY: CDS
 (B) LOCATION: complement (106..816)

(ix) CHARACTERISTICS:

(A) NAME/KEY: CDS

(B) LOCATION: 1144..1416

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

10

ATTTACTATG TTATGTTCTG AGGGGAGTGA AAATTCCTTCT AATTCGATGA AGATTCTTGC 60
15 TCAATTGTTA TCAGCTATGC GCCGACCAGA ACACCTTGCC GATCAGCCAA ACGTCTCTTC 120
AGGCCACTGA CTAGCGATAA CTTTCCCCAC AACGGAACAA CTCTCATTGC ATGGGATCAT 180
20 TGGGTACTGT GGGTTTAGTG GTTGTAAGAA CACCTGACCG CTATCCCTGA TCAGTTTCTT 240
GAAGGTAAAC TCATCACCCC CAAGTCTGGC TATGCAGAAA TCACCTGGCT CAACAGCCTG 300
CTCAGGGTCA ACGAGAATTA ACATTCCGTC AGGAAAGCTT GGCTTGGAGC CTGTTGGTGC 360
25 GGTCAATGAA TTACCTTCAA CCTCAAGCCA GAATGCAGAA TCACTGGCTT TTTTGGTTGT 420
GCTTACCCAT CTCTCCGCAT CACCTTTGGT AAAGGTTCTA AGCTTAGGTG AGAACATCCC 480
30 TGCCTGAACA TGAGAAAAAA CAGGGTACTC ATACTCACTT CTAAGTGACG GCTGCATACT 540
AACCGCTTCA TACATCTCGT AGATTTCTCT GCGGATTGAA GGGCTAAATT CTTCAACGCT 600
AACTTTGAGA ATTTTGTAA GCAATGCGGC GTTATAAGCA TTTAATGCAT TGATGCCATT 660
35 AAATAAGCA CCAACGCCTG ACTGCCCCAT CCCCATCTTG TCTGCGACAG ATTCTGGGA 720
TAAGCCAAGT TCATTTTCT TTTTTCATA AATTGCTTTA AGGCGACGTG CGTCCTCAAG 780
40 CTGCTCTTGT GTTAATGGTT TCTTTTTTGT GCTCATACGT TAAATCTATC ACCGCAAGGG 840
ATAAATATCT AACACCGCGC GTGTTGACTA TTTTACCTCT GGCGGTGATA ATGGTTGCAT 900
GTACTAAGTA GGTGTATGG AACAAACGCAT AACCTGAAA GATTATGCAA TCGCCTTGG 960
45 GCAAACCAAG ACAGCTAAAG ATCCTCTAGA GTCGACCTGC AGGCATGCAA GCTTATCGAA 1020
TTCTCATTCA GGCTTCTGCC GTTTTGGATT TAACCGAAGA TGATTTGAT TTTCTGACGA 1080
50 GTAACAAAGT TTGGATTGCT ACTGACCGCT CTCGTGCTCG TCGCTGCGTT GAGGCTTGCG 1140
TTT ATG GTA CGC TGG ACT TTG TGG GAT ACC CTC GCT TTC CTG CTC CTG 1188
Met Val Arg Trp Leu Trp Asp Thr Ala Phe Leu Leu Leu
1 5 10 15
55 TTG AGT TTA TTG CTG CCG TCA TTG CTT ATT ATG TTC ATC CCG TCA ACA 1236
Leu Ser Leu Leu Leu Pro Ser Leu Leu Ile Met Phe Ile Pro Ser Thr
20 25 30
60 TTC AAA CGG CCT GTC TCA TCA TGG AAG GCG CTG AAT TTA CGG AAA ACA 1284
Phe Lys Arg Pro Val Ser Ser Trp Lys Ala Leu Asn Leu Arg Lys Thr
35 40 45

65

20

25

(B) TYPE: amino acid

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

35	Met 1	Ser	Thr	Lys	Lys 5	Lys	Pro	Leu	Thr	Gln 10	Glu	Gln	Leu	Glu	Asp 15	Ala
	Arg	Arg	Leu	Lys 20	Ala	Ile	Tyr	Glu	Lys 25	Lys	Lys	Asn	Glu	Leu 30	Gly	Leu
40	Ser	Gln	Glu 35	Ser	Val	Ala	Asp	Lys 40	Met	Gly	Met	Gly	Gln 45	Ser	Gly	Val
45	Gly 50	Ala	Leu	Phe	Asn	Gly	Ile 55	Asn	Ala	Leu	Asn	Ala 60	Tyr	Asn	Ala	Ala
	Leu 65	Leu	Thr	Lys	Ile	Leu 70	Lys	Val	Ser	Val	Glu 75	Glu	Phe	Ser	Pro	Ser 80
50	Ile	Ala	Arg	Glu	Ile 85	Tyr	Glu	Met	Tyr	Glu 90	Ala	Val	Ser	Met	Gln 95	Pro
	Ser	Leu	Arg 100	Ser	Glu	Tyr	Glu	Tyr	Pro 105	Val	Phe	Ser	His 110	Val	Gln	Ala
55	Gly	Met	Phe 115	Ser	Pro	Lys	Leu	Arg 120	Thr	Phe	Thr	Lys	Gly 125	Asp	Ala	Glu
	Arg	Trp 130	Val	Ser	Thr	Thr	Lys 135	Lys	Ala	Ser	Asp	Ser 140	Ala	Phe	Trp	Leu
60	Glu 145	Val	Glu	Gly	Asn	Ser 150	Met	Thr	Ala	Pro	Thr 155	Gly	Ser	Lys	Pro	Ser 160

Phe Pro Asp Gly Met Leu Ile Leu Val Asp Pro Glu Gln Ala Val Glu
165 170 175
5 Pro Gly Asp Phe Cys Ile Ala Arg Leu Gly Gly Asp Glu Phe Thr Phe
180 185 190
Lys Lys Leu Ile Arg Asp Ser Gly Gln Val Phe Leu Gln Pro Leu Asn
195 200 205
10 Pro Gln Tyr Pro Met Ile Pro Cys Asn Glu Ser Cys Ser Val Val Gly
210 215 220
Lys Val Ile Ala Ser Gln Trp Pro Glu Glu Thr Phe Gly
225 230 235
15

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 91 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: protein

- 25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

30 Met Val Arg Trp Thr Leu Trp Asp Thr Leu Ala Phe Leu Leu Leu Leu
1 5 10 15
35 Ser Leu Leu Leu Pro Ser Leu Leu Ile Met Phe Ile Pro Ser Thr Phe
20 25 30
Lys Arg Pro Val Ser Ser Trp Lys Ala Leu Asn Leu Arg Lys Thr Leu
35 40 45
40 Leu Met Ala Ser Ser Val Arg Leu Lys Pro Leu Asn Cys Ser Arg Leu
50 55 60
45 Pro Cys Val Tyr Ala Gln Glu Thr Leu Thr Phe Leu Leu Thr Gln Lys
65 70 75 80
Lys Thr Cys Val Lys Asn Tyr Val Gln Lys Glu
85 90

50

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- 55 (A) LENGTH: 2834 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: double strand
(D) TOPOLOGY: both

60

(vi) INITIAL ORIGIN:
(A) ORGANISM: pCSJ fragment

5 (xi) CHARACTERISTICS:
(A) NAME/KEY: CDS
(B) LOCATION: complement (106..816)

10 (ix) CHARACTERISTICS:
(A) NAME/KEY: CDS
(B) LOCATION: 1025..2104

15 (ix) CHARACTERISTICS:
(A) NAME/KEY: CDS
(B) LOCATION: 2377..2649

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

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 ATTTACTATG TTATGTTCTG AGGGGAGTGA AAATTCCCCT AATTGCATGA AGATTCTTGC 60
 25 TCAATTGTTA TCAGCTATGC GCCGACCAGA ACACCTTGCC GATCAGCCAA ACGTCTCTTC 120
 AGGC TACTGA CTAGCGATAA CTTTCCCCAC AACGGAACAA CTCTCATTGC ATGGGATCAT 180
 TGGGTACTGT GGGTTTAGTG GTTGTAAGAAA CACCTGACCG CTATCCCTGA TCAGTTTCTT 240
 30 GAAGGTAAAC TCATCACCCC CAAGTCTGGC TATGCAGAAA TCACCTGGCT CAACAGCCTG 300
 CTCAGGGTCA ACGAGAATTA ACATTCCGTC AGGAAAGCTT GGCTTGGAGC CTGTTGGTGC 360
 35 GGTCAATGAA TTACCTTCAA CCTCAAGCCA GAATGCAGAA TCACTGGCTT TTTTGGTTGT 420
 GCTTACCCAT CTCTCCGCAT CACCTTTGGT AAAGGTTCTA AGCTTAGGTG AGAACATCCC 480
 TGCCTGAACA TGAGAAAAAA CAGGGTACTC ATACTCACTT CTAAGTGACG GCTGCATACT 540
 40 AACCGCTTCA TACATCTCGT AGATTTCTCT GGCGATTGAA GGGCTAAATT CTTCAACGCT 600
 AACTTTGAGA ATTTTGTAA GCAATGCGGC GTTATAAGCA TTTAATGCAT TGATGCCATT 660
 45 AAATAAAGCA CCAACGCCTG ACTGCCCCAT CCCCATCTTG TCTGCGACAG ATTCTTGGGA 720
 TAAGCCAAGT TCATTTTCTT TTTTTCATA AATTGCTTTA AGGCGACGTG CGTCCTCAAG 780
 CTGCTCTTGT GTTAATGGTT TCTTTTTTGT GCTCATACGT TAAATCTATC ACCGCAAGGG 840
 50 ATAAATATCT AACACGCGC GTGTTGACTA TTTTACCTCT GGCGGTGATA ATGGTTGCAT 900
 GTACTAAGTA GGTTGTATGG AACAACGCAT AACCTGAAA GATTATGCAA TCGCCTTTGG 960
 55 GCAAACCAAG ACAGCTAAAG ATCCTCTAGA GCGCCCGGAA GAGAGTCAAT TCAGGGTGGT 1020
 GAAT GTG AAA CCA GTA ACG TTA TAC GAT GTC GCA GAG TAT GCC GGT GTC 1069
 Val Lys Pro Val Thr Leu Tyr Asp Val Ala Glu Tyr Ala Gly Val
 95 100 105
 60 TCT TAT CAG ACC GTT TCC CGC GTG GTG AAC CAG GCC AGC CAC GTT TCT 1117
 Ser Tyr Gln Thr Val Ser Arg Val Val Asn Gln Ala Ser His Val Ser
 110 115 120

	GC	AAA	ACG	CGG	GAA	AAA	GTG	GAA	GC	GC	ATG	GC	GAG	CTG	AAT	TAC	1165
	Ala	Lys	Thr	Arg	Glu	Lys	Val	Glu	Ala	Ala	Met	Ala	Glu	Leu	Asn	Tyr	
			125					130					135				
5	ATT	CCC	AAC	CGC	GTG	GCA	CAA	CAA	CTG	GC	GGC	AAA	CAG	TCG	TTG	CTG	1213
	Ile	Pro	Asn	Arg	Val	Ala	Gln	Gln	Leu	Ala	Gly	Lys	Gln	Ser	Leu	Leu	
		140					145					150					
	ATT	GGC	GTT	GCC	ACC	TCC	AGT	CTG	GCC	CTG	CAC	GC	CCG	TCG	CAA	ATT	1261
10	Ile	Gly	Val	Ala	Thr	Ser	Ser	Leu	Ala	Leu	His	Ala	Pro	Ser	Gln	Ile	
	155					160					165					170	
	GTC	GC	GC	ATT	AAA	TCT	CGC	GCC	GAT	CAA	CTG	GGT	GCC	AGC	GTG	GTG	1309
15	Val	Ala	Ala	Ile	Lys	Ser	Arg	Ala	Asp	Gln	Leu	Gly	Ala	Ser	Val	Val	
				175						180					185		
	GTG	TCG	ATG	GTA	GAA	CGA	AGC	GGC	GTC	GAA	GCC	TGT	AAA	GC	GC	GTG	1357
	Val	Ser	Met	Val	Glu	Arg	Ser	Gly	Val	Glu	Ala	Cys	Lys	Ala	Ala	Val	
				190					195					200			
20	CAC	AAT	CTT	CTC	GC	CAA	CGC	GTC	AGT	GGG	CTG	ATC	ATT	AAC	TAT	CCG	1405
	His	Asn	Leu	Leu	Ala	Gln	Arg	Val	Ser	Gly	Leu	Ile	Ile	Asn	Tyr	Pro	
			205					210					215				
25	CTG	GAT	GAC	CAG	GAT	GCC	ATT	GCT	GTG	GAA	GCT	GCC	TGC	ACT	AAT	GTT	1453
	Leu	Asp	Asp	Gln	Asp	Ala	Ile	Ala	Val	Glu	Ala	Ala	Cys	Thr	Asn	Val	
		220					225					230					
	CCG	GC	TTA	TTT	CTT	GAT	GTC	TCT	GAC	CAG	ACA	CCC	ATC	AAC	AGT	ATT	1501
30	Pro	Ala	Leu	Phe	Leu	Asp	Val	Ser	Asp	Gln	Thr	Pro	Ile	Asn	Ser	Ile	
	235					240					245					250	
	ATT	TTC	TCC	CAT	GAA	GAC	GGT	ACG	CGA	CTG	GGC	GTG	GAG	CAT	CTG	GTC	1549
35	Ile	Phe	Ser	His	Glu	Asp	Gly	Thr	Arg	Leu	Gly	Val	Glu	His	Leu	Val	
				255						260					265		
	GCA	TTG	GGT	CAC	CAG	CAA	ATC	GC	CTG	TTA	GC	GGC	CCA	TTA	AGT	TCT	1597
	Ala	Leu	Gly	His	Gln	Gln	Ile	Ala	Leu	Leu	Ala	Gly	Pro	Leu	Ser	Ser	
				270					275					280			
40	GTC	TCG	GC	CGT	CTG	CGT	CTG	GCT	GGC	TGG	CAT	AAA	TAT	CTC	ACT	CGC	1645
	Val	Ser	Ala	Arg	Leu	Arg	Leu	Ala	Gly	Trp	His	Lys	Tyr	Leu	Thr	Arg	
			285					290					295				
45	AAT	CAA	ATT	CAG	CCG	ATA	GC	GAA	CGG	GAA	GGC	GAC	TGG	AGT	GCC	ATG	1693
	Asn	Gln	Ile	Gln	Pro	Ile	Ala	Glu	Arg	Glu	Gly	Asp	Trp	Ser	Ala	Met	
		300					305					310					
	TCC	GGT	TTT	CAA	CAA	ACC	ATG	CAA	ATG	CTG	AAT	GAG	GGC	ATC	GTT	CCC	1741
50	Ser	Gly	Phe	Gln	Gln	Thr	Met	Gln	Met	Leu	Asn	Glu	Gly	Ile	Val	Pro	
	315					320					325					330	
	ACT	GC	ATG	CTG	GTT	GCC	AAC	GAT	CAG	ATG	GC	CTG	GGC	GCA	ATG	CGC	1789
55	Thr	Ala	Met	Leu	Val	Ala	Asn	Asp	Gln	Met	Ala	Leu	Gly	Ala	Met	Arg	
				335						340					345		
	GCC	ATT	ACC	GAG	TCC	GGG	CTG	CGC	GTT	GGT	GC	GAT	ATC	TCG	GTA	GTG	1837
	Ala	Ile	Thr	Glu	Ser	Gly	Leu	Arg	Val	Gly	Ala	Asp	Ile	Ser	Val	Val	
				350					355					360			
60	GGA	TAC	GAC	GAT	ACC	GAA	GAC	AGC	TCA	TGT	TAT	ATC	CCG	CCG	TCA	ACC	1885
	Gly	Tyr	Asp	Asp	Thr	Glu	Asp	Ser	Ser	Cys	Tyr	Ile	Pro	Pro	Ser	Thr	
			365					370					375				
65	ACC	ATC	AAA	CAG	GAT	TTT	CGC	CTG	CTG	GGG	CAA	ACC	AGC	GTG	GAC	CGC	1933
	Thr	Ile	Lys	Gln	Asp	Phe	Arg	Leu	Leu	Gly	Gln	Thr	Ser	Val	Asp	Arg	
		380					385					390					

	TTG CTG CAA CTC TCT CAG GGC CAG GCG GTG AAG GGC AAT CAG CTG TTG	1981
	Leu Leu Gln Leu Ser Gln Gly Gln Ala Val Lys Gly Asn Gln Leu Leu	
	395 400 405 410	
5	CCC GTC TCA CTG GTG AAA AGA AAA ACC ACC CTG GCG CCC AAT ACG CAA	2029
	Pro Val Ser Leu Val Lys Arg Lys Thr Thr Leu Ala Pro Asn Thr Gln	
	415 420 425	
	ACC GCC TCT CCC CGC GCG TTG GCC GAT TCA TTA ATG CAG CTG GCA CGA	2077
10	Thr Ala Ser Pro Arg Ala Leu Ala Asp Ser Leu Met Gln Leu Ala Arg	
	430 435 440	
	CAG GTT TCC CGA CTG GAA AGC GGG CAG TGAGCGCAAC GCAATTAATG	2124
15	Gln Val Ser Arg Leu Glu Ser Gly Gln	
	445 450	
	TGAGTTAGCT CACTCATTAG GCACCCCAGG CTTTACACTT TATGCTTCCG GCTCGTATGT	2184
	TGTGTGGAAT TGTGAGCGGA TAACAATTTT ACACAGGAAA CAGCTCTGCA GGCATGCAAG	2244
20	CTTATCGAAT TCTCATTCAG GCTTCTGCCG TTTTGGATTT AACC GAAGAT GATTTCGATT	2304
	TTCTGACGAG TAACAAAGTT TGGATTGCTA CTGACCGCTC TCGTGCTCGT CGCTGCGTTG	2364
25	AGGCTTGCGT TT ATG GTA CGC TGG ACT TTG TGG GAT ACC CTC GCT TTC	2412
	Met Val Arg Trp Thr Leu Trp Asp Thr Leu Ala Phe	
	1 5 10	
	CTG CTC CTG TTG AGT TTA TTG CTG CCG TCA TTG CTT ATT ATG TTC ATC	2460
30	Leu Leu Leu Leu Ser Leu Leu Leu Pro Ser Leu Leu Ile Met Phe Ile	
	15 20 25	
	CCG TCA ACA TTC AAA CGG CCT GTC TCA TCA TGG AAG GCG CTG AAT TTA	2508
35	Pro Ser Thr Phe Lys Arg Pro Val Ser Ser Trp Lys Ala Leu Asn Leu	
	30 35 40	
	CGG AAA ACA TTA TTA ATG GCG TCG AGC GTC CGG TTA AAG CCG CTG AAT	2556
40	Arg Lys Thr Leu Leu Met Ala Ser Ser Val Arg Leu Lys Pro Leu Asn	
	45 50 55 60	
	TGT TCG CGT TTA CCT TGC GTG TAC GCG CAG GAA ACA CTG ACG TTC TTA	2604
	Cys Ser Arg Leu Pro Cys Val Tyr Ala Gln Glu Thr Leu Thr Phe Leu	
	65 70 75	
45	CTG ACG CAG AAG AAA ACG TGC GTC AAA AAT TAC GTG CAG AAG GAG	2649
	Leu Thr Gln Lys Lys Thr Cys Val Lys Asn Tyr Val Gln Lys Glu	
	80 85 90	
	TGATGTAATG TCTAAAGGTA AAAACGTTT TGGCGCTCGC CCTGGTCGTC CGCAGCCGTT	2709
50	GCGAGGTACT AAAGGCAAGC GTAAAGGCGC TCGTCTTTGG TATGTAGGTG GTCAACAATT	2769
	TTAATTGCAG GGGCTTCGGC CCTTACTTGA GGATAAATTA TGTCTAATAT TCAAACCTGGC	2829
55	GCCGA	2834

60 (2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 237 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

65

(ii) TYPE OF MOLECULE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

5 Met Ser Thr Lys Lys Lys Pro Leu Thr Gln Glu Gln Leu Glu Asp Ala
1 5 10 15
Arg Arg Leu Lys Ala Ile Tyr Glu Lys Lys Lys Asn Glu Leu Gly Leu
20 25 30
10 Ser Gln Glu Ser Val Ala Asp Lys Met Gly Met Gly Gln Ser Gly Val
35 40 45
Gly Ala Leu Phe Asn Gly Ile Asn Ala Leu Asn Ala Tyr Asn Ala Ala
15 50 55 60
Leu Leu Thr Lys Ile Leu Lys Val Ser Val Glu Glu Phe Ser Pro Ser
65 70 75 80
20 Ile Ala Arg Glu Ile Tyr Glu Met Tyr Glu Ala Val Ser Met Gln Pro
85 90 95
Ser Leu Arg Ser Glu Tyr Glu Tyr Pro Val Phe Ser His Val Gln Ala
100 105 110
25 Gly Met Phe Ser Pro Lys Leu Arg Thr Phe Thr Lys Gly Asp Ala Glu
115 120 125
Arg Trp Val Ser Thr Thr Lys Lys Ala Ser Asp Ser Ala Phe Trp Leu
30 130 135 140
Glu Val Glu Gly Asn Ser Met Thr Ala Pro Thr Gly Ser Lys Pro Ser
145 150 155 160
35 Phe Pro Asp Gly Met Leu Ile Leu Val Asp Pro Glu Gln Ala Val Glu
165 170 175
Pro Gly Asp Phe Cys Ile Ala Arg Leu Gly Gly Asp Glu Phe Thr Phe
180 185 190
40 Lys Lys Leu Ile Arg Asp Ser Gly Gln Val Phe Leu Gln Pro Leu Asn
195 200 205
Pro Gln Tyr Pro Met Ile Pro Cys Asn Glu Ser Cys Ser Val Val Gly
45 210 215 220
Lys Val Ile Ala Ser Gln Trp Pro Glu Glu Thr Phe Gly
225 230 235
50

(2) INFORMATION FOR SEQ ID NO: 9:

55 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 360 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

60 (ii) TYPE OF MOLECULE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

5 Val Lys Pro Val Thr Leu Tyr Asp Val Ala Glu Tyr Ala Gly Val Ser
 1 5 10 15
 Tyr Gln Thr Val Ser Arg Val Val Asn Gln Ala Ser His Val Ser Ala
 20 25 30
 10 Lys Thr Arg Glu Lys Val Glu Ala Ala Met Ala Glu Leu Asn Tyr Ile
 35 40 45
 Pro Asn Arg Val Ala Gln Gln Leu Ala Gly Lys Gln Ser Leu Leu Ile
 50 55 60
 15 Gly Val Ala Thr Ser Ser Leu Ala Leu His Ala Pro Ser Gln Ile Val
 65 70 75 80
 Ala Ala Ile Lys Ser Arg Ala Asp Gln Leu Gly Ala Ser Val Val Val
 85 90 95
 20 Ser Met Val Glu Arg Ser Gly Val Glu Ala Cys Lys Ala Ala Val His
 100 105 110
 25 Asn Leu Leu Ala Gln Arg Val Ser Gly Leu Ile Ile Asn Tyr Pro Leu
 115 120 125
 Asp Asp Gln Asp Ala Ile Ala Val Glu Ala Ala Cys Thr Asn Val Pro
 130 135 140
 30 Ala Leu Phe Leu Asp Val Ser Asp Gln Thr Pro Ile Asn Ser Ile Ile
 145 150 155 160
 Phe Ser His Glu Asp Gly Thr Arg Leu Gly Val Glu His Leu Val Ala
 165 170 175
 35 Leu Gly His Gln Gln Ile Ala Leu Leu Ala Gly Pro Leu Ser Ser Val
 180 185 190
 40 Ser Ala Arg Leu Arg Leu Ala Gly Trp His Lys Tyr Leu Thr Arg Asn
 195 200 205
 Gln Ile Gln Pro Ile Ala Glu Arg Glu Gly Asp Trp Ser Ala Met Ser
 210 215 220
 45 Gly Phe Gln Gln Thr Met Gln Met Leu Asn Glu Gly Ile Val Pro Thr
 225 230 235 240
 Ala Met Leu Val Ala Asn Asp Gln Met Ala Leu Gly Ala Met Arg Ala
 245 250 255
 50 Ile Thr Glu Ser Gly Leu Arg Val Gly Ala Asp Ile Ser Val Val Gly
 260 265 270
 55 Tyr Asp Asp Thr Glu Asp Ser Ser Cys Tyr Ile Pro Pro Ser Thr Thr
 275 280 285
 Ile Lys Gln Asp Phe Arg Leu Leu Gly Gln Thr Ser Val Asp Arg Leu
 290 295 300
 60 Leu Gln Leu Ser Gln Gly Gln Ala Val Lys Gly Asn Gln Leu Leu Pro
 305 310 315 320
 Val Ser Leu Val Lys Arg Lys Thr Thr Leu Ala Pro Asn Thr Gln Thr
 325 330 335

Ala Ser Pro Arg Ala Leu Ala Asp Ser Leu Met Gln Leu Ala Arg Gln
340 345 350

5 Val Ser Arg Leu Glu Ser Gly Gln
355 360

10 (2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 91 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

15

(ii) TYPE OF MOLECULE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

20

Met Val Arg Trp Thr Leu Trp Asp Thr Leu Ala Phe Leu Leu Leu Leu
1 5 10 15

25

Ser Leu Leu Leu Pro Ser Leu Leu Ile Met Phe Ile Pro Ser Thr Phe
20 25 30

30

Lys Arg Pro Val Ser Ser Trp Lys Ala Leu Asn Leu Arg Lys Thr Leu
35 40 45

Leu Met Ala Ser Ser Val Arg Leu Lys Pro Leu Asn Cys Ser Arg Leu
50 55 60

35

Pro Cys Val Tyr Ala Gln Glu Thr Leu Thr Phe Leu Leu Thr Gln Lys
65 70 75 80

Lys Thr Cys Val Lys Asn Tyr Val Gln Lys Glu
85 90

40

Claims

1. Method for selecting mutated O_R or O_L operator DNA sequences from lambdoid phages which have a different thermostability compared to the wild-type sequence with regard to binding a repressor,
wherein
 - (a) a DNA cassette is prepared which contains a selection gene under the operative control of an expression control sequence comprising at least one O_R or O_L operator sequence from a lambdoid phage and a promoter,
 - (b) the operator DNA sequence is subjected to a mutagenesis and
 - (c) the mutated operator DNA sequences are analysed.
2. Method as claimed in claim 1,
wherein

the lambdoid phages are selected from the group comprising the phage lambda, phage 21, phage 22, phage 82, phage 424, phage 434, phage D326, phage DLP12, phage gamma, phage HK022, phage P4, phage Phi80, phage Phi81, coliphage 186 and recombinant variants thereof.
3. Method as claimed in claim 2,
wherein

the phage lambda or recombinant variants thereof are used.

4. Method as claimed in claim 3,
wherein
an operator DNA sequence from the operator regions O_R or/and O_L of the phage lambda is used.
5. Method as claimed in one of the claims 1 - 4,
wherein
the E-lysis gene from the phage PhiX174 is used as the selection gene.
6. Method as claimed in one of the claims 1 - 5,
wherein
the operator DNA sequence is subjected to a site-specific mutagenesis by oligonucleotides or a selection is carried out in a mutator bacterial strain.
7. Method as claimed in one of the claims 1 - 6,
wherein
the mutated operator DNA sequences are analysed by determining their ability to bind to a temperature-sensitive cI repressor.
8. Method as claimed in claim 7,
wherein
the temperature-sensitive lambda repressor cI857 is used.
9. Mutated O_R or O_L operator sequences from lambdoid phages which have a different thermostability compared to the wild-type sequence with regard to binding of a repressor and are obtainable by a method as claimed in one of the claims 1 - 8.

10. Mutated O_R or O_L operator sequences from lambdoid phages which have an increased thermostability compared to the wild-type sequence with regard to binding of a temperature-sensitive repressor and are obtainable by a method as claimed in one of the claims 1 - 8.
11. Mutated O_R or O_L operator sequence as claimed in claim 10,
wherein
it has an approximately 3 - 10°C increased thermostability.
12. Mutated O_R or O_L operator sequence as claimed in claim 10,
wherein
it has an approximately 7 - 9°C increased thermostability.
13. Mutated lambda O_R or O_L operator sequence as claimed in one of the claims 9 - 12, which is a variant of the sequences shown in SEQ ID NO.1 or SEQ ID NO.3.
14. Mutated lambda O_R operator sequence comprising the sequence shown in SEQ ID NO.2.
15. Use of a mutated O_R or O_L operator sequence as claimed in one of the claims 9 - 14 for the temperature-regulated expression of genes in bacterial cells.

16. Use of a combination of (a) a wild-type O_R or O_L operator region and at least one operator region which contains a mutated O_R or O_L operator sequence as claimed in one of the claims 9 - 14 or (b) several operator regions which contain mutated O_R or O_L operator sequences as claimed in one of the claims 9 - 14 with different thermostabilities for the temperature-regulated sequential expression of genes.
17. Use as claimed in claim 15 or 16,
wherein
the bacterial cells contain a gene for a cI repressor from lambdoid phages for the regulation of gene expression.
18. Use as claimed in claim 17,
wherein
the bacterial cells contain the gene for the lambda $cI857$ repressor.
19. Nucleic acid comprising a bacterial expression control sequence which contains a mutated O_R or O_L operator sequence as claimed in one of the claims 9 - 14 in operative linkage with a protein-coding sequence.
20. Nucleic acid as claimed in claim 19,
wherein
the protein-coding sequence is a suicide gene.
21. Nucleic acid as claimed in claim 20,
wherein
the expression control sequence contains a lambda P_L or P_R promoter.

22. Vector,
wherein
it contains at least one copy of a nucleic acid as
claimed in one of the claims 19 - 21.
23. Vector as claimed in claim 22,
wherein
it is a bacterial chromosomal vector.
24. Vector as claimed in claim 22,
wherein
it is a bacterial extrachromosomal plasmid.
25. Bacterial cell,
wherein
it is transformed with a nucleic acid as claimed in
one of the claims 19 - 21 or with a vector as
claimed in one of the claims 22 - 24.
26. Bacterial cell as claimed in claim 25,
wherein
it contains the nucleic acid or the vector
integrated into its chromosome.
27. Bacterial cell as claimed in claim 25 or 26,
wherein
it additionally contains a gene for a cI repressor
from lambdoid phages.
28. Bacterial cell as claimed in claim 27,
wherein
it contains the gene for the lambda cI857
repressor.

29. Vaccine composition,

wherein

it contains a live bacterial cell as claimed in one of the claims 26 - 28 as an active ingredient optionally with pharmaceutically acceptable auxiliary substances, additives and carrier substances.

30. Vaccine composition,

wherein

it contains a bacterial ghost as the active ingredient optionally with pharmaceutically acceptable auxiliary substances, additives and carrier substances in which the bacterial ghost can be obtained by culturing a bacterial cell as claimed in one of the claims 25 - 28 at temperatures of 35 - 39°C and subsequently lysing the bacterial cell by increasing the temperature.

31. Nucleic acid comprising (a) a first bacterial expression control sequence which contains an O_R or O_L operator sequence from a lambdoid phage and to which a first cI repressor from lambdoid phages can bind, in operative linkage with a sequence coding for a second repressor wherein the second repressor cannot bind to the first bacterial expression sequence and (b) a second bacterial expression control sequence to which the second repressor can bind in operative linkage with a suicide gene.

32. Bacterial cell,

wherein

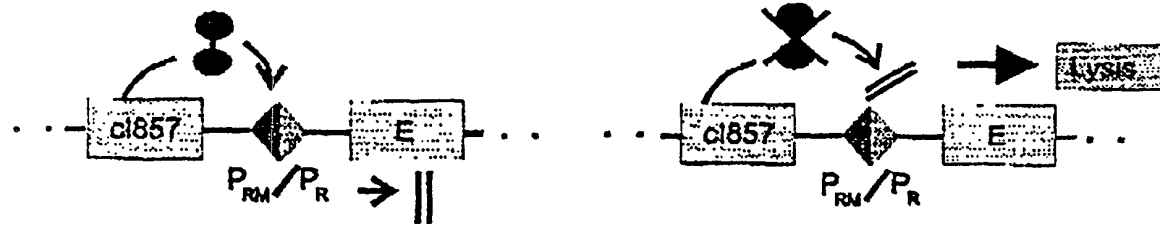
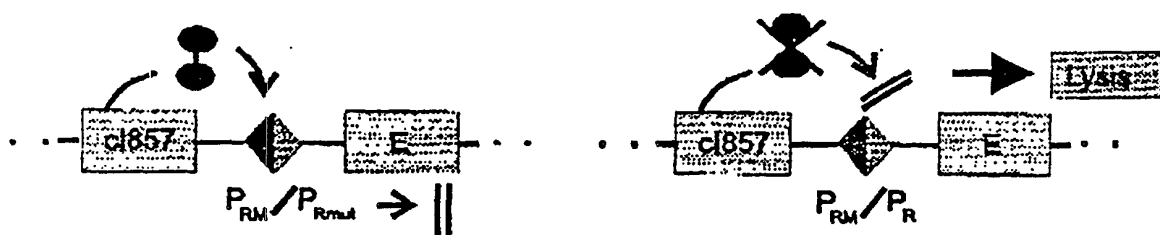
it contains at least one copy of a nucleic acid as claimed in claim 31.

33. Bacterial cell as claimed in claim 32,
wherein
it additionally contains a gene for the first
repressor.
34. Bacterial cell as claimed in claim 32 or 33,
wherein
it contains the first bacterial expression control
sequence of a mutated operator sequence as claimed
in one of the claims 9 - 14.
35. Bacterial cell as claimed in one of the claims 32 -
34 additionally comprising (c) a third bacterial
expression control sequence which contains a
mutated operator sequence as claimed in one of the
claims 9 - 14 in operative linkage with a suicide
gene.
36. Vaccine composition,
wherein
it contains a live bacterial cell as claimed in one
of the claims 32 - 35 as the active ingredient
optionally together with pharmaceutically
acceptable auxiliary substances, additives and
carrier substances.
37. Use of vaccine compositions as claimed in claim 29
or 36 as heat-sensitive or/and cold-sensitive safe
live vaccines.

Abstract

The present invention concerns a method for selecting new P_R or P_L operator sequences from lambdoid phages which have a different thermostability compared to the wild-type sequence with regard to binding a repressor. In addition new mutated P_R or P_L operator sequences and their use for the temperature-regulated expression of genes and for production of improved vaccines is disclosed.

1/5

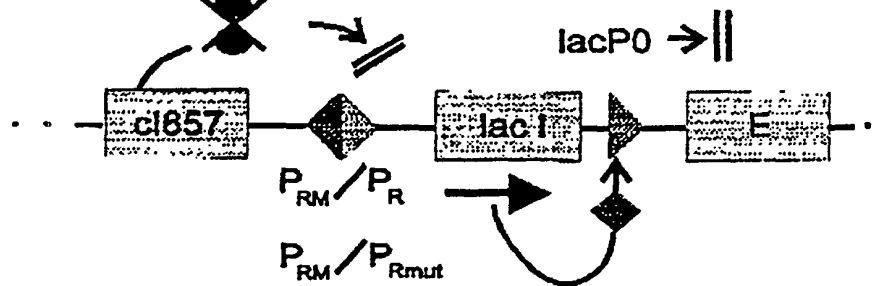
1a) pAW12 $\leq 30^{\circ}\text{C}$  $\geq 30^{\circ}\text{C}$ b) pAWJ12 $\leq 37^{\circ}\text{C}$  $\geq 39^{\circ}\text{C}$

2a)

 $\geq 32^{\circ}\text{C}$

pCS1

pCSJ1

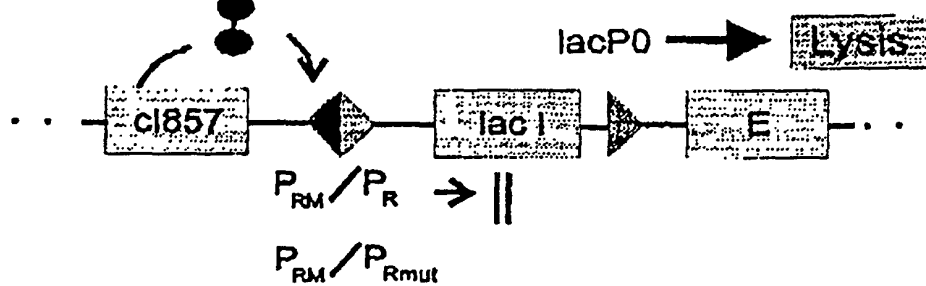


2b)

 $\leq 30^{\circ}\text{C}$

pCS1

pCSJ1



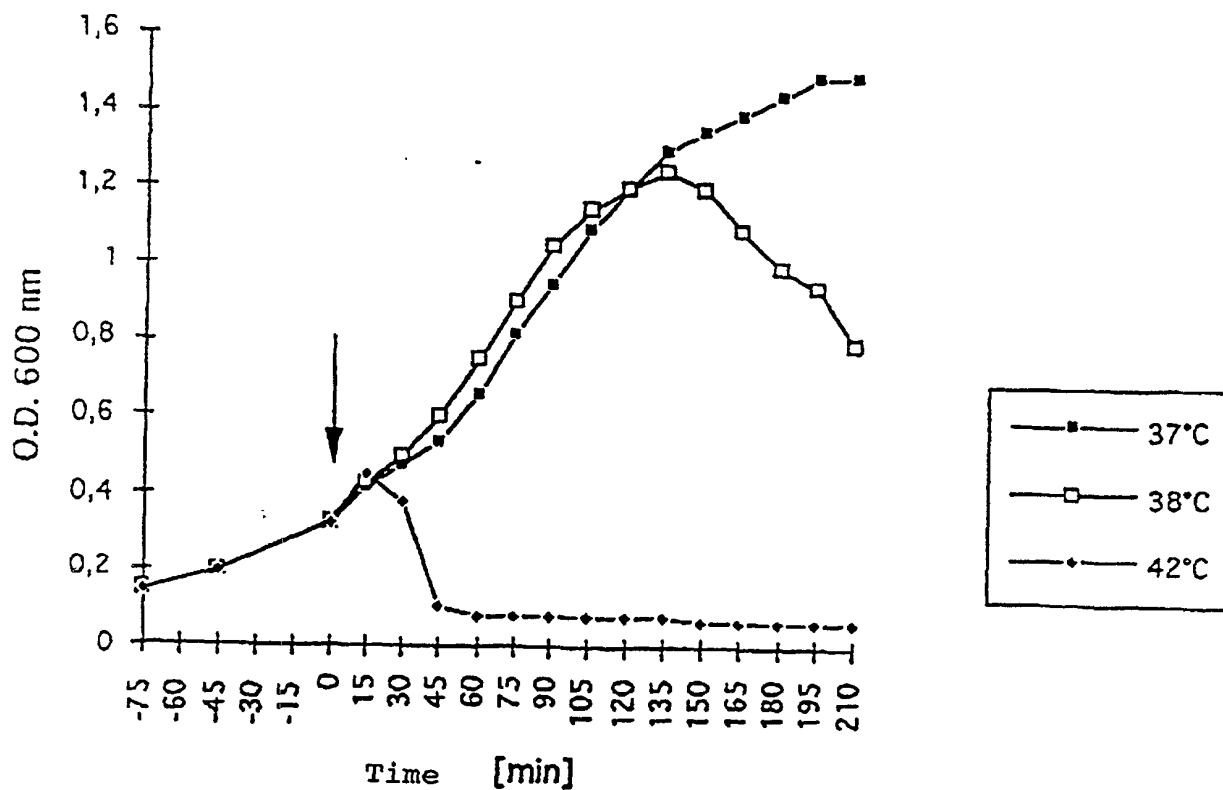


Fig. 3: Growth of *E. coli* NM522 (pAWJ12) when the temperature is changed from 28°C to higher temperatures (↓)

3/5

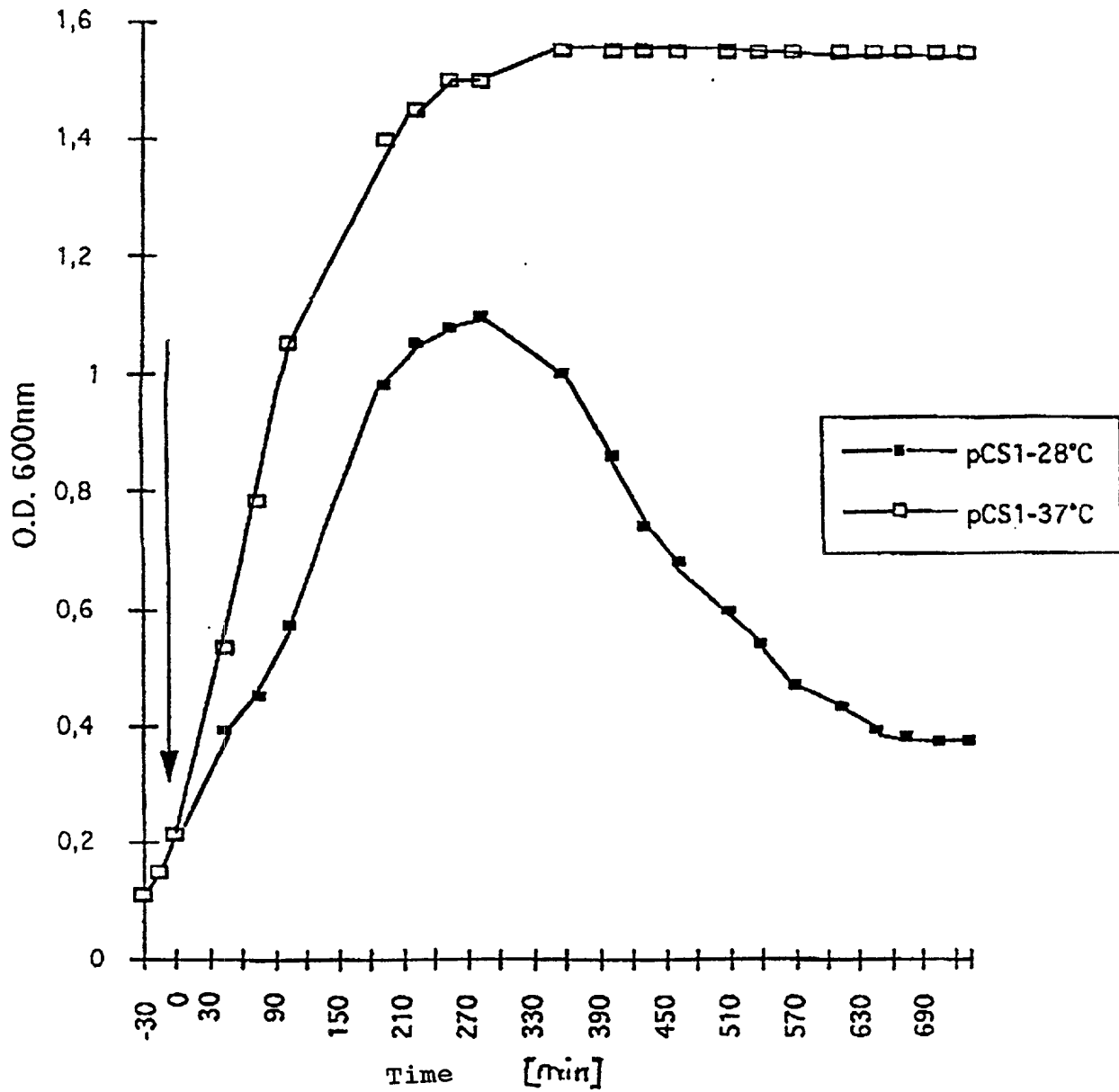


Fig. 4: Growth of *E. coli* MC4100 (pCS1) when the temperature is changed from 37°C to 28°C (↓)

4/5

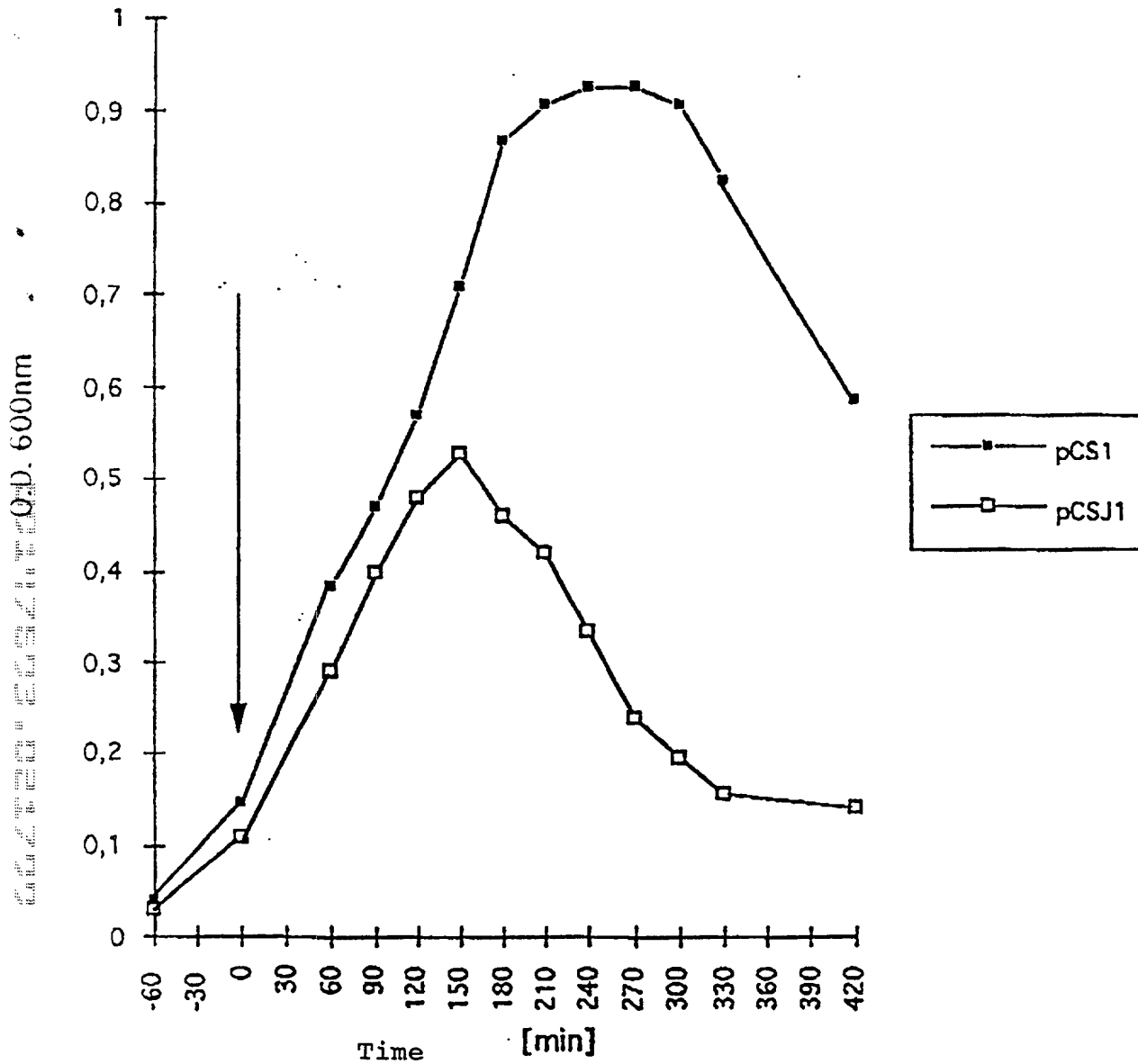


Fig. 5: Growth of *E. coli* MC4100 (pCS1) and MC4100 (pCSJ1) when the temperature is changed from 37°C to 28°C (↓)

N, M, M & O Docket No. _____

NIKAIDO, MARMELESTEIN, MURRAY & ORAM

Declaration For U.S. Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention **entitled** (Insert Title) New systems for the regulation of gene expression

the specification of which

- (Check one of blocks 1, 2 or 3. See note A on back of this page)
1. ☐ is attached hereto.
 2. ☒ was filed on August 21, 1997 as International PCT Application Serial No. PCT/EP 97/04560 and was amended on _____ (if applicable)
 3. ☐ was filed on _____ as U.S. Application Serial No. _____ and was amended on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application for which priority is claimed:

(List prior foreign applications. See note B on back of this page)	<u>196 33 698.8</u> (Number)	<u>DE</u> (Country)	<u>Aug. 21, 1996</u> (Day/Month/Year Filed)	Priority Claimed <input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
	_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	<input type="checkbox"/> Yes <input type="checkbox"/> No
	_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	<input type="checkbox"/> Yes <input type="checkbox"/> No
	_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	<input type="checkbox"/> Yes <input type="checkbox"/> No

(See Note C on back of this page)

☐ See attached list for additional prior foreign applications

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT International filing date of this application:

(List prior U.S. Applications or PCT International applications designating the U.S.)	(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
	_____ (Application Serial No.)	_____ (Filing Date)	_____ (Status) (patented, pending, abandoned)

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(See Note D on back of this page)

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Inventor's signature A. Witte 19.1.1999
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Citizenship German
Post Office Address same as above

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Inventor's signature _____ Date _____
Residence _____
Citizenship _____
Post Office Address _____

Full name of sixth joint inventor, if any _____
Inventor's signature _____ Date _____
Residence _____
Citizenship _____
Post Office Address _____

Full name of seventh joint inventor, if any _____
Inventor's signature _____ Date _____
Residence _____
Citizenship _____
Post Office Address _____

Full name of eighth joint inventor, if any _____
Inventor's signature _____ Date _____
Residence _____
Citizenship _____
Post Office Address _____